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ORIGINAL PAPER

Immunolocalization of β- and δ-giardin within the ventral disk in trophozoites of *Giardia duodenalis* using multiplex laser scanning confocal microscopy

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Abstract Immunolocalization of β- and δ-giardin in *Giardia duodenalis* trophozoites revealed that both giardins are strictly associated with the ventral disk (VD). Optical sectioning of the immunolabeled VD, together with quantitative colocalization of δ- and β-giardin immunoreactivity, demonstrated that δ-giardin is primarily localized to the ventral side, and β-giardin is localized to the dorsal side of the VD.

Introduction

Giardia duodenalis is a binucleate protozoan parasite that colonizes the upper small intestine of humans and animals causing diarrheal disease. The study of *Giardia* biology and efforts to develop control strategies against the parasite have been greatly advanced by the complete sequencing of its genome (Morrison et al. 2007). To maintain infection within the small intestine, trophozoites, the replicative stage of the parasite, must attach to the epithelial layer of the gut and resist its peristaltic movement, bolus flow, and continuous shedding of mucus and cells. Unlike other Diplomonadida, *Giardia* has a unique organelle, the ventral disk (VD, Elmendorf et al. 2003), which is believed to play a key role in *Giardia* attachment to host epithelial cells (Palm et al. 2005). The rigid structure of the VD, also referred to as the adhesive or sucking disk, is supported by a spiral array of microtubules emanating from posterior flagellar bodies. Adjacent microtubules are connected to microribbons and cross-bridges. In addition to highly conserved tubulin and actin components, the VD is composed of proteins, identified as α-, β-, γ-, and δ-giardins (Nohria et al. 1992). Of those, definite localization within the VD has been shown only for β-giardin, also referred to as the striated fiber assemblin homolog (Crossley and Holberton 1985; Elmendorf et al. 2003; Holberton and Ward 1981; Holberton et al. 1988). Although giardins are components of the cytoskeleton, there are reports that β-giardin in *Giardia muris* can also be expressed on the trophozoite surface (Heyworth et al. 1999) or associated with the VD membrane in the bare zone (Palm et al. 2005). Original conflicting evidence on the location of α-giardins (the annexin homologs) has been resolved by the discovery of 21 different α-giardins found associated with flagella, the VD, or on the trophozoite surface (Weiland et al. 2005). It appears certain that α1- and α2-giardins are associated with the plasma membrane, and play some role in attachment of trophozoites to host cells (Aggarwal and Nash 1989; Wenman et al. 1993; Weiland et al. 2003). Current knowledge of γ- and δ-giardin localization in the VD is limited. In recent studies it was shown that antirecombinant δ-giardin antibodies preferably recognized the VD and inhibited trophozoite binding to an inanimate surface (Jenkins et al. 2009). The present study was undertaken to provide novel information on the relative location of δ- and β-giardins within the *G. duodenalis* VD.

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Materials and methods

Cells *G. duodenalis* (WB strain, assemblage A) trophozoites were cultured in modified TYI-S-33 medium (Keister 1983).

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RT-PCR, cloning, and purification of giardins Cloning and purification of recombinant δ-giardin was conducted as described (Jenkins et al. 2009). Recombinant β-giardin was produced by first amplifying the respective complementary DNA (cDNA) by RT-PCR using primers (Table 1) derived from DNA sequences for β-giardin (X07919, ORF-frame 3). In brief, 1 ng of total RNA was subjected to real-time RT-PCR using 1 pmol of β-forward and reverse primers, and the Superscript III One-Step RT-PCR system (Invitrogen, Carlsbad, California) in a 25-μl reaction volume. RT-PCR consisted of reverse transcription at 47°C for 1 h, denaturation at 94°C for 1 min, followed by 35 cycles of 94°C for 30 s, 59°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5 min. RT-PCR products were purified using a PCR purification kit (Qiagen, Valencia, CA), and eluates were ethanol precipitated, dried at room temperature, and suspended in 10 μl of sterile H_2O, followed by KpnI and HindIII digestion using standard procedures (Sambrook et al. 1989). The expression vector pTrcHisB (Invitrogen) was also digested with the identical enzymes. After digestion at 37°C for 2 h, both β-giardin RT-PCR product and pTrcHisB were subjected to agarose electrophoresis, visualized by EtBr staining, excised from the gel, and purified using a gel purification kit (Qiagen) and ligated overnight at 15°C using T4 DNA ligase (New England Biolabs, Ipswich, MA). The ligation mixtures were transformed in *Escherichia coli* DH5 cells according to standard procedures (Hanahan 1983), and recombinant β-giardin clones were identified by colony PCR (Güssow and Clackson 1989) using pTrcHis-specific primers (pTrcHis-forward 5′ CTG TAC GAC GAT GAC GAT AAG 3′ and pTrcHis-reverse 5′ TCA TCC GCC AAA ACA GCC AAG). The reading frame and orientation of the cDNAs were confirmed by DNA sequencing of at least three recombinant plasmids using a pTrcHis-universal primer (5′ CGATTAAATAAGGAGG 3′) and a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA). Sequencing reactions were run on an ABI3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA), analyzed using Sequencher 4.9 software (GeneCodes Corp., Ann Arbor, MI)

For high level expression, recombinant pTrcHis-β giardin plasmid DNA was transformed into *E. coli* BL21 (Novagen, San Diego, CA). Cultures were grown in Luria-Bertani medium containing 100 μg/ml ampicillin (Sigma Chemical Co., St. Louis, MO) at 37°C until the O.D. reached 0.5, whereupon expression of recombinant β-giardin was induced by the presence of 1 mM isopropylthiocyanate (IPTG, Sigma). The cultures were harvested after 4 h IPTG induction by centrifugation at 3,000×*g* for 10 min. The cell pellets were extracted with native binding buffer (Invitrogen, Carlsbad, CA) containing phenylmethylsulfonyl fluoride protease inhibitor (Sigma), frozen–thawed two times between a dry-ice ethanol bath and a 37°C water bath, and sonicated twice for 15 s each, with incubation on wet ice for 1 min between sonications. The protein extracts were treated with 1 U/ml RNase and DNase for 30 min at room temperature and pelleted by centrifugation at 5,000×*g* for 30 min. The insoluble pellet was extracted by suspension in denaturing binding buffer (Invitrogen) for 30 min at room temperature on a rocker. The extracts were pelleted by centrifugation at 5,000×*g* for 30 min, and the supernatant subjected to NiNTA affinity chromatography to purify recombinant β-giardin protein.

Table 1 Primer sequences used in the cloning of cDNA coding for *G. duodenalis* β-giardin protein

Primer name	Primer sequence (5′–3′)
Beta-forward	AATTTGGTACCATGGACAAGCCCGACGA
Beta-reverse	GGGTTCGAATTAGTGCTTTGTACC

Underlined sequence refers to restriction enzyme recognition site
Beta-forward KpnI, *Beta-reverse* HindIII

Preparation of antisera Polyclonal sera specific for β-giardin was prepared by Pacific Immunology, Inc. (Ramona, CA) by immunizing two New Zealand white rabbits with 100 μg/injection of NiNTA-purified recombinant β-giardin emulsified in complete Freunds adjuvant (CFA, primary immunization) or incomplete Freunds adjuvant (ICFA, three booster immunizations). Polyclonal sera specific for δ-giardin was prepared by the same company (Pacific Immunology) by immunizing 1 goat with 200 μg/injection of NiNTA-purified recombinant δ-giardin in CFA (primary immunization) or ICFA (three booster immunizations).

Immunoblotting analysis Recombinant δ- or β-giardin protein was fractionated by SDS-polyacrylamide gel electrophoresis followed by transblotting to Immobilon membrane (Millipore, Billerica, MA) in a semidry transblotter apparatus (BioRad, Hercules, CA). After transfer, the membranes were treated with phosphate-buffered saline (PBS) containing 2% nonfat dry milk (PBS-NFDM) to block nonspecific immunoglobulin binding in subsequent steps. After blocking, the membranes were incubated with either rabbit anti-recombinant β-giardin or goat anti-δ-giardin sera or antisera to an irrelevant polyHis fusion protein (1:1,000 dilution) for 2–4 h at room temperature (RT) on a laboratory shaker, followed by 2 h incubation with biotinylated goat antirabbit IgG or biotinylated rabbit antigoat IgG (1:1,000 dilution) (Sigma), and 1 h with avidin peroxidase (Sigma, 1:5,000 dilution). All antibodies were diluted in PBS containing 0.05% Tween 20 (PBS-TW), and removed after each step by three washes with PBS-TW. Binding of anti-giardin antibodies was assessed by a final incubation with peroxidase substrat—0.5 mg/ml 4-chloro-1-napthol (Sigma) and 0.015% H_2O_2 (Sigma) in PBS.

Immunofluorescence staining Prior to immunofluorescence staining, *G. duodenalis* trophozoites were treated with paraformaldehyde (PF) to retain integrity of the plasma membrane (El-Kon et al. 2009). *G. duodenalis* trophozoites were treated in with 3% PF in PBS for 5 min at RT, followed by three washes with PBS, and treatment for 30 min with 2% PBS-NFDM to reduce nonspecific binding in subsequent steps. Staining was accomplished by incubating 10^4 trophozoites for 1 h in a 100-μl suspension of 1:1,000 dilution of rabbit antirecombinant β-giardin or goat anti-δ-giardin sera followed by a 1-h incubation with 100 μl fluorescein isothiocyanate (FITC) goat antirabbit IgG (Sigma) or Alexa Fluor 633-conjugated donkey antigoat IgG (Invitrogen) diluted to 20 μg/ml. Antibodies were removed between incubations by washing trophozoites three times in PBS followed by centrifugation for 1 min at 3,000×*g*. Approximately 10^4 trophozoites were pipetted in individual wells of multiwell slides (Erie Scientifc Co., Portsmouth, NH) and then overlaid with 5 μl/well of Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and a glass coverslip.

Immunofluorescence staining of permeabilized trophozoites was conducted by first pipetting 10^4 trophozoites in individual wells of multiwell slides (Erie Scientifc Co., Portsmouth, NH) and allowing the parasites to air-dry at RT for 1 h. The slides were then either used directly or treated with methanol for 5 min followed by gentle rinsing in PBS. After drying, the multiwell slides containing trophozoites were stored at −70°C. Just prior to immunofluorescence staining of trophozoites, the slides were removed from the freezer and allowed to warm to RT. Nonspecific binding of antibodies was blocked by the treatment of each well with PBS-NFDM for 30 min at RT. The slides were gently rinsed with PBS, allowed to air-dry, and then incubated singly or in combination with a 1:1,000 dilution of goat anti-δ-giardin sera or rabbit anti-β-giardin sera. The slides were incubated in a humidified chamber for 1 h at RT, and then gently rinsed three times with PBS and allowed to air-dry, followed by staining with either FITC-conjugated rat antirabbit IgG (Sigma) or Alexa Fluor 633-conjugated donkey antigoat IgG (Invitrogen). In order to avoid cross-reactivity of secondary antibodies, Alexa Fluor 633-conjugated donkey antigoat IgG was applied in one step, and the FITC goat antirabbit IgG was applied in a second step. To observe if Alexa Flour 633 or FITC spectral properties can affect spatial distribution of giardins immunoreactivity, a dye swap experiment was conducted wherein the FITC mouse antigoat IgG (Sigma) was followed by Alexa Fluor 633-conjugated goat antirabbit IgG (Invitrogen) after incubation with primary anti-δ-giardin and anti-β-giardin sera. After the final incubation, the slides were gently rinsed with PBS, allowed to air-dry, and then overlaid with 5 μl/well of Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and a glass coverslip.

A Zeiss 710 laser scanning confocal microscopy (LSCM) system was utilized in immunolocalization analysis. The images were observed using a Zeiss Axio Observer inverted microscope with ×63 and ×100 1.4 NA oil immersion Plan Apochromatic objectives. A photomultiplier tube captured in a single-track mode the specimen fluorescence excited by: (1) a 488-nm green laser and emitted fluorescence passing through an FITC filter with limits set between 510 and 535 nm, for detection of FITC-conjugated antibodies or (2) a 633-nm red laser passing through an MBS-488/561/633 filter with limits set between 638 and 747 nm for detection of Alexa Fluor 633-conjugated antibodies. Zeiss Zen™ 2008 software was used to obtain the images with 1,024×1,024- and 2,048×2,048-pixel resolution and Z stacks ranging from 22 to 60 focal planes. Zeiss AxioVision version 4.8.3 with 4D software was used to construct three-dimensional (3D) images of

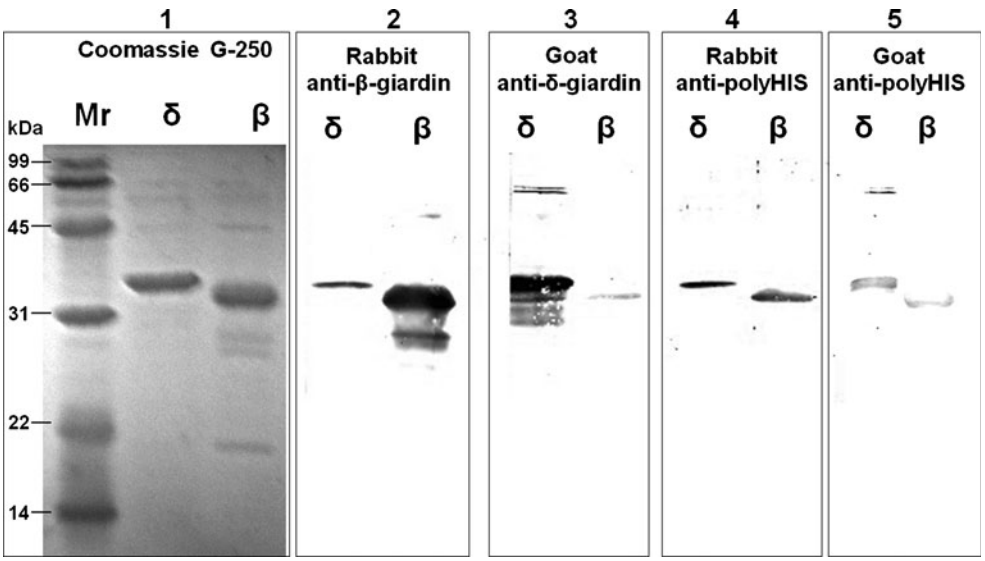


Fig. 1 SDS-PAGE/immunoblotting analysis of recombinant β- and δ-giardins. *Panel 1* Coomassie Blue staining, *panels 2–5* immunostaining with specific antisera. *Panel 2* rabbit anti-β-giardin, *panel 3* goat anti-δ-giardin, *panel 4* rabbit anti-irrelevant polyHis protein, *panel 5* goat anti-irrelevant polyHis protein

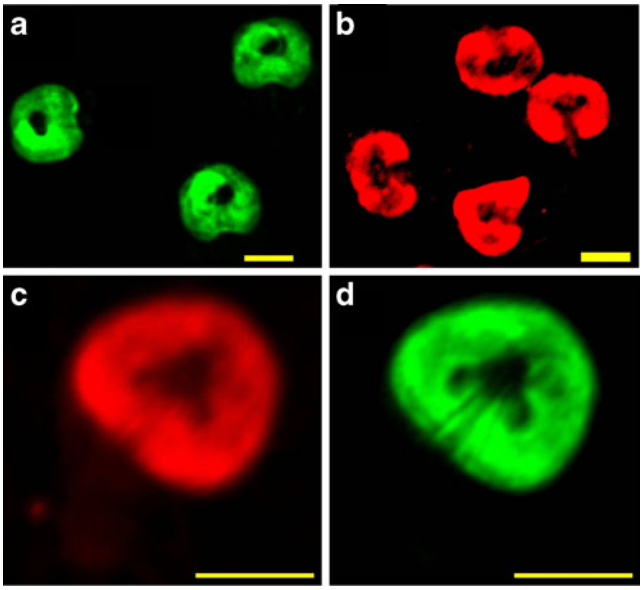


Fig. 2 Fluorescence microscopy images of methanol-fixed *G. duodenalis* trophozoites singly labeled with antibodies against β- and δ-giardin. **a** Antibodies to recombinant β-giardin followed by FITC (*green*)-labeled secondary antibodies; **b** antibodies to recombinant δ-giardin followed by Alexa Fluor 633 (*red*)-conjugated secondary antibodies. **c**, **d** Images of a permeabilized trophozoite double labeled with antibodies against recombinant δ- and β-giardin respectively. *Scale bar* 5 μm

specimens. Colocalization module integrated in Zeiss Zen™ 2008 software was used to generate scatter plots and calculate colocalization coefficients.

Results and discussion

Immunoblotting analysis of goat anti-δ-giardin or rabbit anti-β-giardin sera showed that polyclonal antibodies specifically recognized the corresponding recombinant giardin

Fig. 4 Colocalization analysis of β- and δ-giardin within seven representative optical sections of *G. duodenalis* trophozoites. Optical sectioning was conducted from the ventral side (*top panels*) to the dorsal side (*bottom panels*) of the trophozoite. *Red channel* and *green channel* fluorescence images show the level of immunoreactivity for δ- and β-giardin, respectively. Multichannel (*red* and *green* overlay) fluorescence images show colocalization of δ- and β -giardins with yellow pixels. Only pixels within the outlined area were used in quantitative colocalization. Scatter plots show the distribution of florescence signal (immunoreactivity) between two channels together with the number of colocalized pixels (area 3) for every optical section. A heat map on the right shows the absolute frequency of pixels in scatter plots. *Scale bar* 5 μm

(Fig. 1). Faint recognition of δ-giardin observed with rabbit anti-β-giardin sera was similar to that observed with rabbit antisera prepared against an irrelevant recombinant polyHis fusion protein (Fig. 1). Also, goat antisera to an irrelevant recombinant polyHis fusion protein showed slight recognition of both recombinant β- and δ-giardins which may reflect binding of a common polyHis portion of recombinant proteins (Fig. 1).

In methanol-fixed, permeabilized trophozoites that were singly labeled and subjected to LSCM analysis, antibodies to recombinant β- and δ-giardin localized to the VD (Fig. 2a, b). In permeabilized trophozoites double labeled with antibodies against recombinant β- and δ-giardin, both antigens appear to be present only in association with the VD, as staining outside of the VD was negligible (Fig. 2c, d). Although slightly more intense, the recognition pattern of β-giardin in nonpermeabilized (PF-fixed) trophozoites was similar to that observed with permeabilized (methanol-fixed) parasites (Fig. 3a–c). However, anti-δ-giardin staining of nonpermeabilized trophozoites produced a more diffuse labeling compared to that observed with permeabilized trophozoites (Fig. 3d–f). While peripheral staining of the VD was observed, δ-giardin appeared to be concentrated

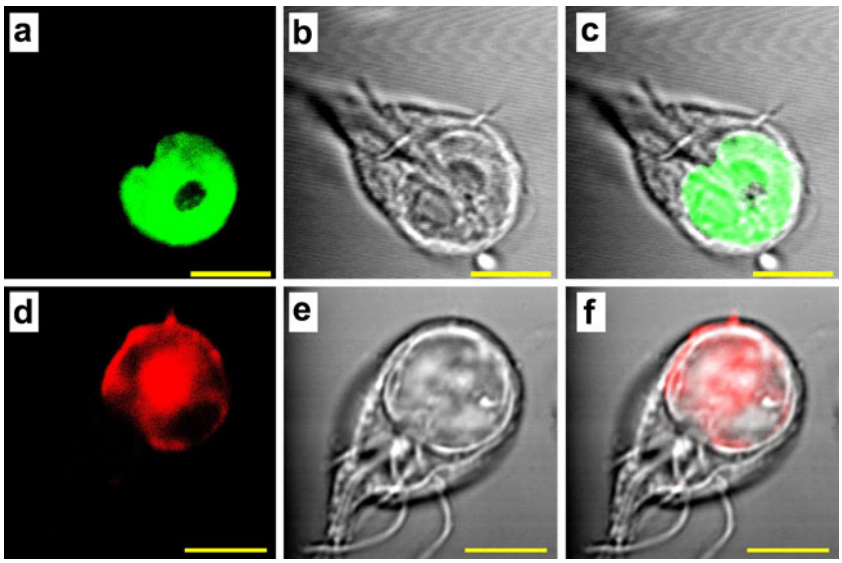
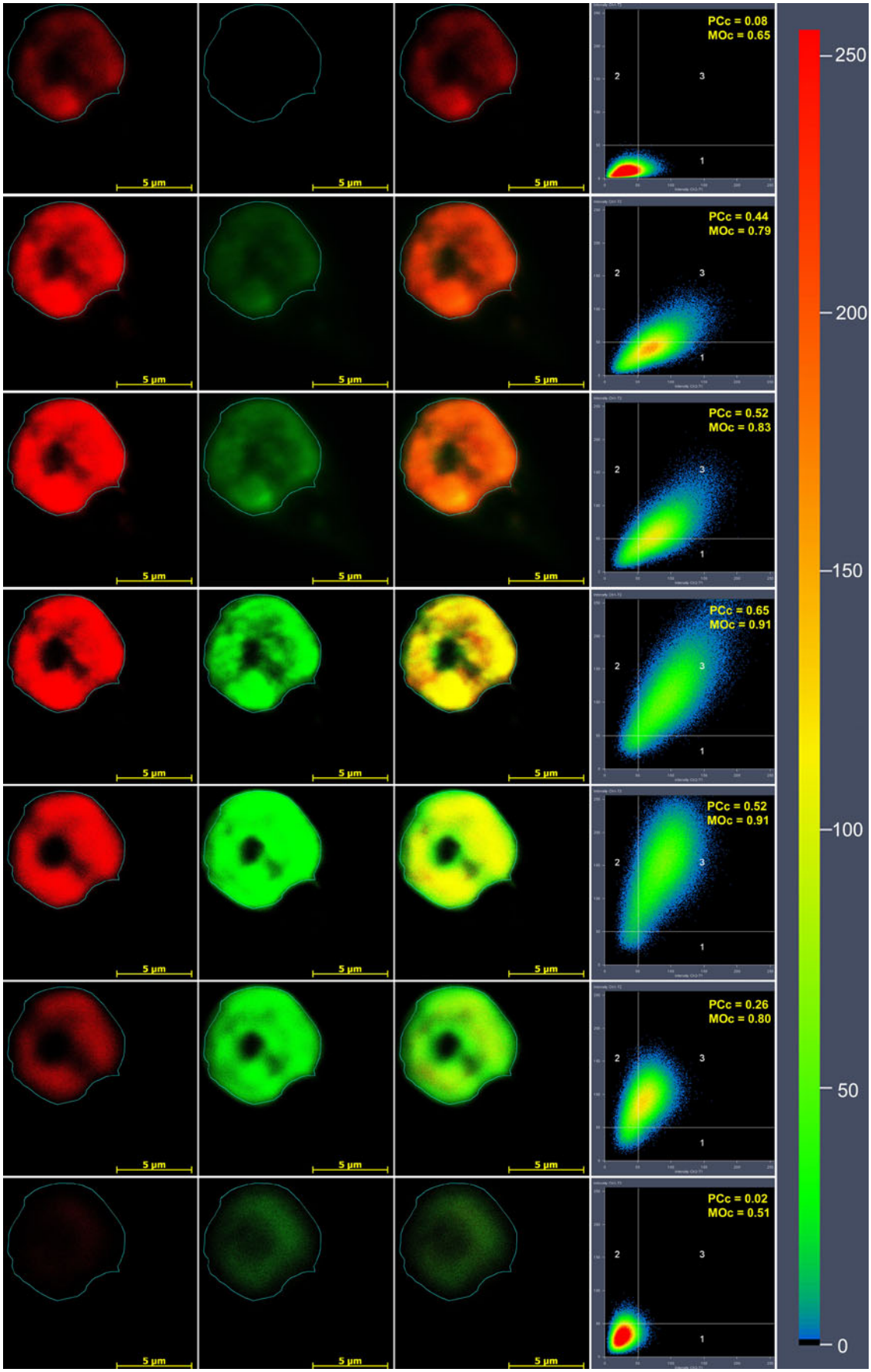


Fig. 3 LSCM images of ventral side of paraformaldehyde-fixed *G. duodenalis* trophozoites labeled with antibodies against β-giardin (**a**–**c**) or δ-giardin (**d**–**f**). **a**, **d** Fluorescence; **b**, **e** differential interference contrast (DIC); **c**, **f** overlay of fluorescence with DIC images. *Scale bar* 5 μm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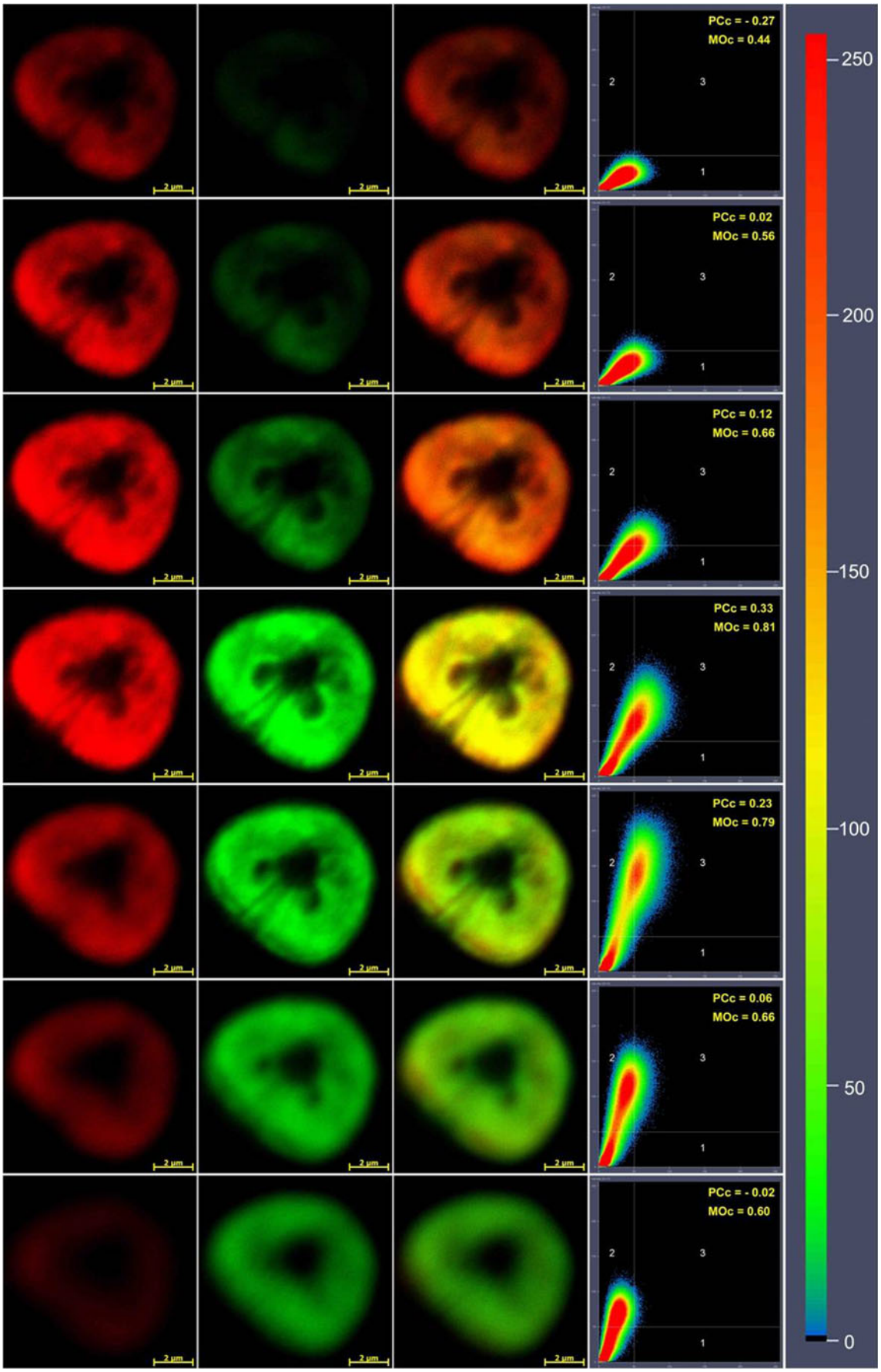
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2 µm
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◀ **Fig. 5** Colocalization analysis of β- and δ-giardins within seven representative optical sections of *G. duodenalis* VD. Optical sectioning was conducted from the ventral side (*top panels*) to the dorsal side (*bottom panels*) of the disk. *Red channel* and *green channel* fluorescence images show the level of immunoreactivity for δ- and β-giardin, respectively. Multichannel (*red* and *green* overlay) fluorescence images show colocalization of δ- and β -giardin with yellow pixels. Scatter plots show the distribution of florescence signal (immunoreactivity) between two channels together with the number of colocalized pixels (area 3) for every optical section. A heat map on the right shows the absolute frequency of pixels in scatter plots. *Scale bar* 2 μm

in the center of the disk in nonpermeabilized trophozoites. These data suggest that both β- and δ-giardin are associated with the plasma membrane.

For in-depth analysis of β- and δ-giardin localization within the VD, methanol-fixed trophozoites were double labeled with antibodies against recombinant β- and δ-giardin, and then subjected to high-resolution scanning along the *Z* axis. Scanning of the multiple focal planes of entire trophozoites or of VD alone identified specific immunoreactivity for each giardin within the VD. Seven representative optical sections (from 54 acquired) of a trophozoite demonstrate different degrees of colocalization of β- and δ-giardin in the VD along the *Z* axis (Fig. 4). Scatter plots show the quantitative distribution of florescence signal (intensity) between two channels (β-giardin green and δ-giardin red) together with the number of colocalized pixels for each optical section presented. The disk areas, in which β- and δ-giardin are colocalized, are also shown in the form of yellow pixels in the two-channel micrographs for every optical section (Fig. 4). The results of the quantitative colocalization as well as single- and multichannel confocal images demonstrate the gradual shift in the intensity of immunoreactivity for β- and δ-giardin (Fig. 4). For δ-giardin, a higher intensity was observed between the midsection and the ventral side of the VD. In contrast, higher intensity of immunoreactivity for β-giardin was localized between the midsection and dorsal side of the VD. The maximum degree of colocalization of β- and δ-giardin was found in the midsection of the VD; this can be observed on the two-channel micrographs as the highest number of yellow pixels, as well as in the scatter plot showing the greatest number of pixels in area no. 3 (Fig. 4). This pattern of β- and δ-giardin distribution within the VD is also confirmed by two independent quantitative parameters of colocalization: *Pearson's correlation* coefficient and *Mander's overlap* coefficient (Adler and Parmryd 2010; Manders et al. 1993). Both parameters acquired the highest values, 0.65 and 0.91, respectively, in the midsection of the VD (Fig. 4). Quantitative colocalization analysis of the higher resolution images (2,048×2,048 pixels), resulting from the optical sectioning of the VD alone, revealed an identical pattern for distribution of β- and δ-giardin along the *Z* axis of the VD, as described above. Seven representative optical sections (from 22 acquired) along with pixel colocalization graphs, and Pearson's and Mander's coefficient values are shown in Fig. 5. Again, stronger intensity of the Alexa633 (δ-giardin) fluorophore is observed in the top (ventral side of the VD) optical sections. In the lower panels (midsection of the disk) Alexa633 and FITC have comparable intensities and a greater degree of colocalization; this is also supported by the scatter plots together with Pearson's correlation and Mander's overlap coefficients values, 0.33 and 0.81, respectively (Fig. 5). Bottom panels that represent optical sections from the dorsal side of the VD illustrate predominantly FITC

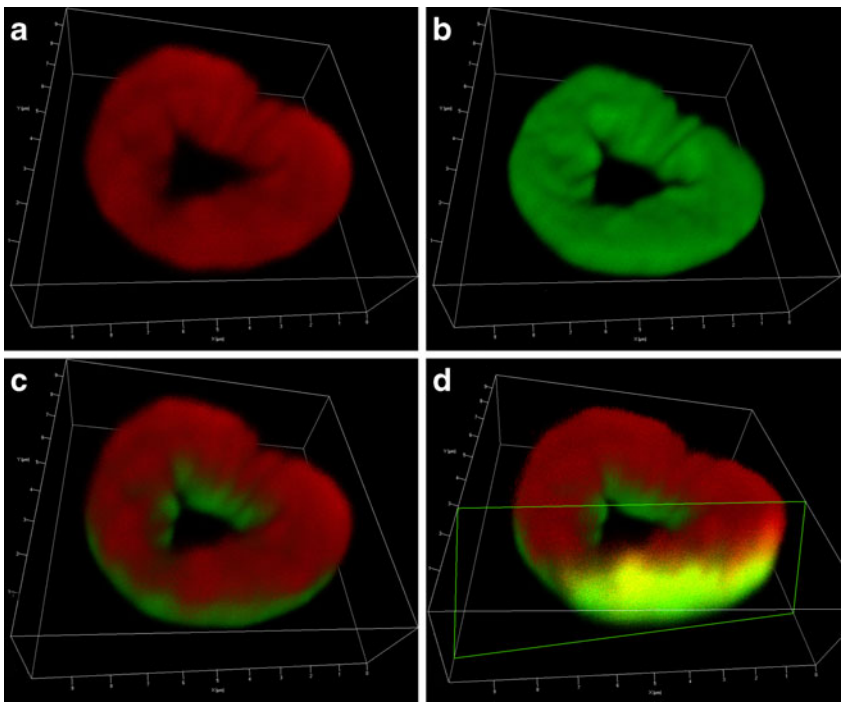

Fig. 6 Three-dimensional projection image of the *G. duodenalis* VD, positioned with the ventral side upwards. **a** Spatial localization of δ-giardin is shown as *red* fluorescence and that of **b** β-giardin as *green* fluorescence. **c** Spatial localization of giardins δ and β. **d** A section through the disk shows δ- and β-giardin colocalization as *yellow* pixels

fluorescence and lower values for quantitative parameters of colocalization.

A 3D representation of the spatial localization of β- and δ-giardins to the VD, positioned with the ventral side upwards, was generated using AxioVision 4-D software (Fig. 6). A 3D distribution of the δ-giardin (red fluorescence) to the VD is shown in panel a, and for β-giardin in panel b. Spatial localization of both δ- and β-giardin within the VD revealed δ-giardin on the ventral side and β-giardin on the dorsal side (Fig. 6c). A section through the disk (panel d) shows δ- and β-giardin colocalization in the form of yellow pixels. An identical localization of β- and δ-giardins was observed in a dye swap experiment when the trophozoites were labeled with FITC-conjugated mouse antigoat IgG (Sigma) and Alexa633-conjugated goat anti-rabbit IgG (H + L; Invitrogen) antibodies (data not shown), demonstrating that distribution of the immunoreactivity for β- and δ-giardins was not affected by the spectral properties of the fluorophores.

In summary, the present immunofluorescence-based localization study is consistent with earlier reports that identified β-giardin associated with the VD (Crossley and Holberton 1985; Holberton and Ward 1981). This study demonstrated for the first time that δ-giardin, even though it shares a great degree of colocalization with β-giardin, is present more ventrally and therefore is closest to the side of the VD that makes contact with host cells. Furthermore, in contrast to a dominating belief that β-giardin associates strictly with the VD, antibodies to β-giardin and δ-giardin were found bound to PF-fixed nonpermeabilized *G. lamblia* trophozoites, indicating that these proteins can be exposed on the surface of the VD as was suggested in previous studies (Heyworth et al. 1999; Palm et al. 2005). Whether β-giardin or δ-giardins can bind to host cells in vitro, as has been observed for alpha-1 giardin (Weiland et al. 2005), remains to be determined.

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